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(54) **Modified pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase with superior substrate specificity and stability**

(57) The present invention relates to modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) having lower activity with respect to disaccharides and/or greater stability than wild-type PQQGDH.

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**Description****BACKGROUND OF THE INVENTION****1. Field of the Invention**

[0001] The present invention relates to a modified glucose dehydrogenase (GDH) with improved substrate specificity and/or thermal stability, and more specifically to a modified PQQ-dependent glucose dehydrogenase (PQQGDH) having pyrroloquinoline quinone (PQQ) as the coenzyme, and to a manufacturing method and a glucose sensor.

[0002] The modified PQQGDH of the present invention is useful for measuring glucose in clinical assay, food analysis and the like.

**2. Description of the Related Art**

[0003] PQQGDH is a glucose dehydrogenase having pyrroloquinoline quinone (PQQ) as its coenzyme. Because PQQGDH catalyzes a reaction in which glucose is oxidized to produce gluconolactone, it can be used in measuring blood sugar. Blood glucose concentration is extremely important in clinical diagnosis as a marker of diabetes. At present, the principal method of measuring blood glucose employs a biosensor using glucose oxidase, but the reaction is affected by dissolved oxygen concentration, raising the possibility of errors in the measurements. Attention has focused on PQQ-dependent glucose dehydrogenase as a substitute for glucose oxidase. Our group discovered that the NCIMB 11517 strain of *Acinetobacter baumannii* produces PQQ-dependent glucose dehydrogenase, conducted gene cloning and constructed a high-expression system (Japanese Patent Application Laid-Open No. H11-243949). Compared with glucose oxidase, PQQ-dependent glucose dehydrogenase had problems in substrate specificity and thermal stability.

[0004] An object of the present invention is to provide a PQQGDH with improved substrate specificity and/or thermal stability.

**BRIEF DESCRIPTION OF THE DRAWINGS****[0005]**

Figure 1 shows measurement results for optimum pH;  
 Figure 2 shows the results of glucose assay;  
 Figure 3 shows results for activity of Q76K on maltose;  
 Figure 4 shows results for activity of Q76E on maltose;  
 Figure 5 shows results for activity of Q168V on maltose;  
 Figure 6 shows results for activity of Q168A on maltose; and  
 Figure 7 shows results for activity of the wild-type enzyme on maltose.

**SUMMARY OF THE INVENTION**

[0006] The present invention provides a modified PQQGDH, a manufacturing method therefor, and a glucose assay kit and glucose sensor containing said PQQGDH.

1. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) having less activity on disaccharides and/or greater stability than wild-type PQQGDH.
2. The modified PQQGDH according to 1, wherein activity on disaccharides is less than that of wild-type glucose dehydrogenase.
3. The modified PQQGDH according to 2, wherein the disaccharide is maltose.
4. The modified PQQGDH according to 2, wherein the activity on maltose is no more than 90% of that on glucose.
5. The modified PQQGDH according to 2, wherein the Km value for disaccharides is increased.
6. The modified PQQGDH according to 5, wherein the disaccharide is maltose.
7. The modified PQQGDH according to 5, wherein the Km value for maltose is 8 mM or greater.
8. The modified PQQGDH according to 2, wherein the Km value for disaccharides is greater than that for glucose.
9. The modified PQQGDH according to 8, wherein the disaccharide is maltose.
10. The modified PQQGDH according to 8, wherein the Km value for maltose is at least 1.5 times the Km value for glucose.
11. The modified PQQGDH according to 2, wherein amino acids involved in glucose binding and/or surrounding

amino acids are substituted in the PQQ-dependent glucose dehydrogenase shown in SEQ ID No. 1.

12. The modified PQQGDH according to 2, wherein one or more amino acids at positions selected from the group consisting of positions 67, 68, 69, 76, 89, 167, 168, 169, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 300, 349, 129, 130 and 131 are substituted in the PQQ-dependent glucose dehydrogenase shown in SEQ ID No. 1.

13. The modified PQQGDH according to 12, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, N167E, N167L, N167G, N167T, N167S, N167A, N167M, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169D, L169S, L169W, L169Y, L169A, L169N, L169M, L169V, L169C, L169Q, L169H, L169F, L169R, L169K, L169I, L169T, K89E, K300R, S207C, N188I, T349S, K300T, L174F, K49N, S189G, F215Y, S189G, E245D, A351T, P67K, E68K, P67D, E68T, I69C, P67R, E68R, E129R, K130G, P131G, E129N, P131T, E129Q, K130T, P131R, E129A, K130K, P131K, E341L, M342P, A343R, A343I, E341P, M342V, E341S, M342I, A343C, M342R, A343N, L169P, L169G and L169E.

14. The modified PQQGDH according to 12, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169A, L169V, L169H, L169K, L169D, L169S, L169N, L169G, L169C, (K89E+K300R), (Q168A+L169D), (Q168S+L169S), (N167E+Q168G+L169T), (N167S+Q168N+L169R), (Q168G+L169T), (N167G+Q168S+L169Y), (N167L+Q168S+L169G), (N167G+Q168S+L169S+L174F+K49N), (Q168N+L168N+S189R), (N167E+Q168G+L169A+S189G), (N167G+Q168R+L169A), (N167S+Q168G+L169A), (N167G+Q168V+L169S), (N167S+Q168V+L169S), (N167T+Q168I+L169G), (N167G+Q168W+L169N), (N167G+Q168S+L169N), (N167G+Q168S+L169V), (Q168R+L169C), (N167S+Q168L+L168G), (Q168C+L169S), (N167T+Q168N+L169K), (N167G+Q168T+L169A+S207C), (N167A+Q168A+L169P), (N167G+Q168S+L169G), (N167G+Q168G), (N167G+Q168D+L169K), (Q168P+L169G), (N167G+Q168N+L169S), (Q168S+L169G), (N188I+T349S), (N167G+Q168G+L169A+F215Y), (N167G+Q168T+L169G), (Q168G+L169V), (N167G+Q168V+L169T), (N167E+Q168N+L169A), (Q168R+L169A), (N167G+Q168R), (N167G+Q168T), (N167G+Q168T+L169Q), (Q168I+L169G+K300T), (N167G+Q168A), (N167T+Q168L+L169K), (N167M+Q168Y+L169G), (N167E+Q168S), (N167G+Q168T+L169V+S189G), (N167G+Q168G+L169C), (N167G+Q168K+L169D), (Q168A+L169D), (Q168S+E245D), (Q168S+L169S), (A351T), (N167S+Q168S+L169S), (Q168I+L169Q), (N167A+Q168S+L169S), (Q168S+L169E), (Q168A+L169G), (Q168S+L169P), (P67K+E68K), (P67R+E68R+I69C), (P67D+E68T+I69C), (E129R+K130G+P131G), (E129Q+K130T+P131R), (E129N+P131T), (E129A+K130R+P131K), (E341L+M342P+A343R), (E341S+M342I), A343I, (E341P+M342V+A343C), (E341P+M342V+A343R), (E341L+M342R+A343N), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169I), (Q168A+L169K), (Q168A+L169M), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169S), (Q168A+L169T), (Q168A+L169V), (Q168A+L169W) and (Q168A+L169Y) to improve substrate specificity.

15. The modified PQQGDH according to 2, wherein an amino acid is inserted between positions 428 and 429 in the PQQ-dependent glucose dehydrogenase shown in SEQ ID No. 1.

16. A gene coding for the modified PQQGDH according to any of 1-15.

17. A vector containing the gene according to 16.

18. A transformant transformed by the vector according to 17.

19. A method of manufacturing a modified PQQGDH, comprising cultivating the transformant according to 18.

20. A glucose assay kit comprising the modified PQQGDH according to any of 1-19.

21. A glucose sensor comprising the modified PQQGDH according to any of 1-19.

22. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) wherein stability is improved over that of wild-type PQQGDH.

23. The modified PQQGDH according to 23, wherein residual activity after heat treatment at 58° C for 30 minutes is 48% or more.

24. The modified PQQGDH according to 23, wherein residual activity after heat treatment at 58° C for 30 minutes is 55% or more.

25. The modified PQQGDH according to 23, wherein residual activity after heat treatment at 58° C for 30 minutes is 70% or more.

26. The modified PQQGDH according to 23, wherein one or more amino acids at positions selected from the group consisting of positions 20, 76, 89, 168, 169, 246 and 300 are substituted in the PQQGDH shown in SEQ ID No. 1.

27. The modified PQQGDH according to 26, wherein the amino acid substitutions are selected from the group of K20E, Q76M, Q76G, K89E, Q168A, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, L169D, L169E, L169P, L169S, Q246H, K300R, Q76N, Q76T, Q76K, L169A, L169C, L169E, L169F, L169H, L169K, L169N, L169Q, L169R, L169T, L169Y and L169G.

28. The modified PQQGDH with improved thermal stability according to 27, wherein the amino acid substitutions are selected from the group of K20E, Q76M, Q76G, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S),

- Q246H, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, (Q168S + L169E), (Q168S + L169P), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169K), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169T), and (Q168A+L169Y) and (Q168A + L169G).
29. A gene encoding the modified PQQGDH according to any of 22-28.
30. A vector comprising the gene according to 29.
31. A transformant transformed by the vector according to 30.
32. A method of manufacturing modified PQQGDH, comprising cultivating the transformant according to 31.
33. A glucose assay kit comprising the modified PQQGDH according to any of 22-31.
34. A glucose sensor comprising the modified PQQGDH according to any of 22-31.
35. A method for determining glucose concentration in a sample using the modified PQQGDH according to any of 22-31.
36. The modified PQQGDH according to 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 15 Å from an active center of three-dimensional active structure of wild type enzyme.
37. The modified PQQGDH according to 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a substrate in a three-dimensional active structure of wild type enzyme-substrate complex.
38. The modified PQQGDH according to 37, whose substrate is glucose.
39. The modified PQQGDH according to 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a OH group bound to a carbon at position 1 of a substrate in a three-dimensional active structure of wild type enzyme-substrate complex.
40. The modified PQQGDH according to 39, whose substrate is glucose.
41. The modified PQQGDH according to 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a OH group bound to a carbon at position 2 of a substrate in a three-dimensional active structure of wild type enzyme-substrate complex.
42. The modified PQQGDH according to 41, whose substrate is glucose.

[0007] In the specification, amino acid positions are numbered beginning with 1 as aspartic acid with the signal sequence removed.

[0008] The modified PQQGDH of the present invention encompasses PQQGDH having less activity on disaccharides and/or greater thermal stability than wild-type PQQGDH.

[0009] Examples of disaccharides include maltose, sucrose, lactose, cellobiose and the like, and particularly maltose.

[0010] Activity on disaccharides signifies the action of dehydrogenating the disaccharides.

[0011] As long as activity on disaccharides is reduced in comparison with wild-type PQQGDH, the modified PQQGDH of the present invention encompasses modified PQQGDH in which the activity on glucose is either increased, unchanged or reduced.

[0012] In the measurement of glucose concentration, the activity on disaccharides of the modified PQQGDH of the present invention, is less than that of the case when wild-type glucose dehydrogenase is used. In the modified PQQGDH of the present invention, the activity on maltose is particularly reduced. Activity on maltose is no more than 90%, preferably 75%, more preferably 60%, particularly 40% of that of wild-type PQQGDH.

[0013] The modified PQQGDH of the present invention may have a greater Km value for disaccharides than for glucose. Modified PQQGDH having a higher Km value for maltose than for glucose is particularly desirable. The Km value for maltose should be at least 8 mM, preferably at least 12 mM, particularly at least 20 mM.

[0014] The modified PQQGDH of the present invention may be further indicated by a higher Km value for disaccharides than for glucose. Modified PQQGDH having a higher Km value for maltose than for glucose is particularly desirable. The Km value for maltose should be at least 1.5 times or preferably at least 3 times that for glucose.

[0015] As used here, "activity on maltose" signifies the ratio of the reaction rate using glucose as a substrate to the reaction rate using disaccharides or specifically maltose as a substrate, expressed as a percentage.

[0016] The degree of improvement of the invention in thermal stability expressed as residual activity after heat treatment at 58° C for 30 minutes is preferably higher than that of wild type PQQGDH. The residual activity of the modified PQQGDH of the invention is at least 48% or preferably 55% or more preferably 70%.

[0017] Examples of the modified PQQGDH with improved substrate specificity of the present invention include GDH having amino acids substituted at one or more of positions 67, 68, 69, 76, 89, 167, 168, 169, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 300, 349, 129, 130 and 131 in the amino acid sequence of SEQ ID No. 1, and GDH having an amino acids inserted between positions 428 and 429. Preferred are GDH having amino acid substitutions selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, N167E, N167L, N167G, N167T, N167S, N167A, N167M, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169D, L169S, L169W, L169Y, L169A, L169N, L169M, L169V, L169C, L169Q,

L169H, L169F, L169R, L169K, L169I, L169T, K89E, K300R, S207C, N188I, T349S, K300T, L174F, K49N, S189G, F215Y, S189G, E245D, A351T, P67K, E68K, P67D, E68T, I69C, P67R, E68R, E129R, K130G, P131G, E129N, P131T, E129Q, K130T, P131R, E129A, K130R, P131K, E341L, M342P, A343R, A343I, E341P, M342V, E341S, M342I, A343C, M342R, A343N, L169P, L169G and L169E, and GDH having L, A or K inserted between positions 428 and 429. Substitutions at positions 67, 68, 69, 76, 89, 167, 168, 169, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 300, 349, 129, 130 and 131 may be at one position or multiple positions.

**[0018]** As used here, "Q76N" signifies the substitution of N (Asn) for Q (Gln) at position 76.

**[0019]** Substitutions of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, Q168I, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169A, L169V, L169H, L169K, L169D, L169S, L169N, L169G, L169C, (K89E+K300R), (Q168A+L169D), (Q168S+L169S), (N167E+Q168G+L169T), (N167S+Q168M+L169R), (Q168G+L169T), (N167G+Q168S+L169Y), (N167L+Q168S+L169G), (N167G+Q168S+L169S+L174F+K49N), (Q168N+L168N+S189R), (N167E+Q168G+L169A+S189G), (N167G+Q168R+L169A), (N167S+Q168G+L169A), (N167G+Q168V+L169S), (N167S+Q168V+L169S), (N167T+Q168I+L169G), (N167G+Q168W+L169N), (N167G+Q168S+L169N), (N167G+Q168S+L169V), (Q168R+L169C), (N167S+Q168L+L168G), (Q168C+L169S), (N167T+Q168N+L169K), (N167G+Q168T+L169A+S207C), (N167A+Q168A+L169P), (N167G+Q168S+L169G), (N167G+Q168G), (N167G+Q168D+L169K), (Q168P+L169G), (N167G+Q168N+L169S), (Q168S+L169G), (M188I+T349S), (N167G+Q168G+L169A+F215Y), (N167G+Q168T+L169G), (Q168G+L169V), (N167G+Q168V+L169T), (N167E+Q168N+L169A), (Q168R+L169A), (N167G+Q168R), (N167G+Q168T), (N167G+Q168T+L169Q), (Q168I+L169G+K300T), (N167G+Q168A), (N167T+Q168L+L169K), (N167M+Q168Y+L169G), (N167E+Q168S), (N167G+Q168T+L169V+S189G), (N167G+Q168G+L169C), (N167G+Q168K+L169D), (Q168A+L169D), (Q168S+E245D), (Q168S+L169S), (A351T), (N167S+Q168S+L169S), (Q168I+L169Q), (N167A+Q168S+L169S), (Q168S+L169E), (Q168A+L169G), (Q168S+L169P), (P67K+E68K), (P67R+E68R+I69C), (P67D+E68T+I69C), (E129R+K130G+P131G), (E129Q+K130T+P131R), (E129N+P131T), (E129A+K130R+P131K), (E341L+M342P+A343R), (E341S+M342I), A343I, (E341P+M342V+A343C), (E341P+M342V+A343R), (E341L+M342R+A343N), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169I), (Q168A+L169K), (Q168A+L169M), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169S), (Q168A+L169T), (Q168A+L169V), (Q168A+L169W) and (Q168A+L169Y), and also insertion of L, A or K between positions 428 and 429 contribute to improving the substrate specificity of PQQGDH. As used here, substrate specificity signifies the ratio (%) of the reaction rate with glucose as the substrate to the reaction rate with disaccharides (particularly maltose) used as the substrate.

**[0020]** The PQQGDH of the present invention with improved thermal stability has amino acid substitutions at one or more of positions 20, 76, 89, 168, 169, 246 and 300 of the amino acid sequence of SEQ ID No. 1, and preferably has amino acid substitutions selected from the group consisting of K20E, Q76M, Q76G, K89E, Q168A, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, L169D, L169E, L169P, L169S, Q246H, K300R, Q76N, Q76T, Q76K, L169A, L169C, L169E, L169F, L169H, L169K, L169N, L169Q, L169R, L169T, L169Y and L169G. Substitutions at positions 20, 76, 89, 168, 169, 246 and 300 may be at one position or multiple positions.

**[0021]** As used here "K20E" signifies that K (Lys) has been replaced with E (Glu) at position 20.

**[0022]** The amino acid substitutions K20E, Q76M, Q76G, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S), Q246H, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, (Q168S + L169E), (Q168S + L169P), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169K), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169T), and (Q168A+L169Y) and (Q168A + L169G) contribute particularly to the thermal stability of the PQQGDH.

**[0023]** The wild-type PQQGDH protein for modification shown in SEQ ID No. 1 and the nucleotide sequence shown in SEQ ID No. 2 are both well known, and are disclosed in Japanese Patent Application Laid-Open No. H11-243949.

**[0024]** There are no particular limits on the method of manufacturing the modified protein of the present invention, which may be manufactured according to the procedures shown below. Commonly used methods of modifying genetic information may be used in modifying the amino acid sequence which constitutes the protein. That is, DNA having the genetic information of the modified protein is created by replacing a specific nucleotide or nucleotides in DNA having the genetic information for the protein, or by inserting or deleting a specific nucleotide or nucleotides. Actual methods of replacing one or more nucleotides in DNA include those employing commercial kits (such as the Clontech Transformer Mutagenesis Kit; Stratagene EXOIII/Mung Bean Deletion Kit; or Stratagene QuickChange Site Directed Mutagenesis Kit), or those employing the polymerase chain reaction (PCR).

**[0025]** The resulting DNA having the genetic information of the modified protein is linked to a plasmid and inserted into a host cell or microorganism to create a transformant which produces the modified protein. Plasmids which can be used include pBluescript, puc18 or the like for example if *Escherichia coli* is the host microorganism. Host organisms which can be used include for example *Escherichia coli* W3110, *Escherichia coli* C600, *Escherichia coli* JM109, *Escherichia coli* DH5 $\alpha$  and the like. Insertion of the vector into the host organism may be accomplished for example by introducing the recombinant DNA in the presence of calcium ions when the host organism is of the genus *Escherichia*,

and the electroporation method may also be used. Commercially available competent cells (such as Toyobo Competent High JM109) can also be used.

[0026] The resulting transformant reliably produces large quantities of the modified protein when cultured in a nutrient medium. The culture conditions for the transformant can be selected according to the physiological requirements of the host; a liquid culture is normally used, but for industrial purposes an aerated agitation culture is advantageous. A wide range of nutrients which are ordinarily used in culturing the organism can be used in the medium. The carbon source may be any convertible carbon compound, including for example glucose, sucrose, lactose, maltose, fructose, molasses and pyruvic acid. The nitrogen source may be any usable nitrogen compound, including for example peptone, meat extract, yeast extract, hydrolyzed casein and soybean meal alkali extract. In addition, phosphates, carbonates, sulfates, salts of magnesium, calcium, potassium, iron, manganese, zinc and the like, specific amino acids and specific vitamins are used as necessary. The culture temperature can be adjusted within the range at which the organism grows and produces the modified protein, but should be about 20-42° C in the case of *Escherichia coli*. The culture time will vary somewhat depending on conditions: the culture can be terminated at a suitable point according to when the maximum yield of the modified protein is obtained, with the normal time range being 6-48 hours. The pH of the culture medium can be varied within the range at which the organism grows and produces the modified protein, with a pH of 6.0-9.0 being particularly desirable.

[0027] Culture medium containing organisms which produce the modified protein can be collected from the culture and used as is, but ordinarily when the modified protein is present in the culture medium a solution containing the modified protein is isolated from the bacterial cells by conventional means such as filtration or centrifugation for purposes of use. When the modified protein is present in the cells, they are harvested from the resulting culture by methods such as filtration or centrifugation, the cells are broken down by mechanical methods or enzymatic methods such as lysozyme, and the modified protein is solubilized as necessary by the addition of a chelating agent such as EDTA and/or a surfactant and isolated and harvested as an aqueous solution.

[0028] The resulting solution containing the modified protein can then be subjected to vacuum concentration, membrane concentration and salting out with ammonium sulfate or sodium sulfate or else precipitation by fractional precipitation using a hydrophilic organic solvent such as methanol, ethanol or acetone. Heat treatment and isoelectric treatment are also effective means of purification. The purified modified protein can be obtained by gel filtration with an adsorbent or gel filter, adsorption chromatography, ion exchange chromatography or affinity chromatography.

[0029] In the present invention, we were able to obtain modified PQQGDH with improved substrate specificity by focusing on the 76, 167, 168 and 169 positions of the PQQGDH shown in SEQ ID No. 1 and creating amino acid substitutions for them. The substitutions Q76K, Q168A, (Q168S + L169S), (Q168A + L169D), (Q168S + E245D), (Q168S + L169E), (Q168A + L169G), (Q168S + L169P), (Q168A + L169A), (Q168A + L169C), (Q168A + L169E), (Q168A + L169K), (Q168A + L169M), (Q168A + L169N), (Q168A + L169P), (Q168A + L169S) and (Q168A + L169T) are particularly desirable in terms of substrate specificity.

[0030] In the present invention, we were able to obtain modified PQQGDH with improved stability by focusing on the 20, 76, 89, 168, 169, 246 and 300 positions of the PQQGDH shown in SEQ ID No. 1 and creating amino acid substitutions for them. The substitutions K20E, (K89E + K300R), Q168A, (Q168A + L169D), (Q168S + L169S), (Q168S + L169E), (Q168S + L169P), (Q168A + L169G), Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168S, Q168W, Q168Y, (Q168A + L169A), (Q168A + L169C), (Q168A + L169E), (Q168A + L169F), (Q168A + L169H), (Q168A + L169K), (Q168A + L169N), (Q168A + L169P), (Q168A + L169Q), (Q168A + L169R), (Q168A + L169T), (Q168A + L169Y) and Q246H are particularly desirable in terms of thermal stability.

#### Glucose Assay Kit

[0031] The present invention also provides a glucose assay kit containing modified PQQGDH according to the present invention. The glucose assay kit of the present invention contains enough modified PQQGDH according to the invention for at least one assay. Typically the kit contains in addition to the modified PQQGDH of the invention such buffers, mediators, standard glucose solutions for making calibration curves, and instructions for use as are needed to perform the assay. The modified PQQGDH according to the invention can be provided in a variety of forms such as for example a freeze-dried reagent or a solution in a suitable storage solution. The modified PQQGDH of the present invention is preferably provided as a holoenzyme, but may also be provided as an apoenzyme and converted at the time of use.

#### Glucose Sensor

[0032] The present invention also provides a glucose sensor which uses modified PQQGDH according to the invention. A carbon, gold or platinum electrode or the like is used as the electrode, with the enzyme of the present invention being immobilized on the electrode. Possible immobilizing methods include those employing crosslinking reagents,

enclosure in a polymer matrix, coating with a dialysis membrane, employing photocrosslinked polymers, conductive polymers, and oxidation-reduction polymers, as well as adsorption fixing on the electrode or fixing in a polymer together with an electron mediator typified by ferrocene and its derivatives, and a combination of these may also be used. Preferably the modified PQQGDH of the present invention is immobilized on the electrode as a holoenzyme, but it may also be immobilized as an apoenzyme. and PQQ supplied in solution or as a separate layer. Typically the modified PQQGDH of the invention is first immobilized on a carbon electrode using glutaraldehyde, and the glutaraldehyde is then blocked by treatment with a reagent having an amine group.

[0033] Glucose concentration can be measured as follows. Buffer is placed in a thermostatic cell and maintained at a constant temperature after the addition of PQQ,  $\text{CaCl}_2$  and a mediator. Mediators that can be used include potassium ferricyanide, phenazine methosulfate and the like. An electrode on which the modified PQQGDH of the present invention has been immobilized is used as the work electrode, together with a counter electrode (such as a platinum electrode) and a reference electrode (such as an Ag/AgCl electrode). A fixed voltage is applied to the carbon electrode, and once the current has stabilized a sample containing glucose is added and the increase in current measured. The glucose concentration in the sample can then be calculated based on a calibration curve prepared from glucose solutions with standard-concentration.

#### BEST MODE FOR CARRYING OUT THE INVENTION

Example 1: Construction of an expression plasmid with PQQ-dependent glucose dehydrogenase gene

[0034] Expression plasmid pNPG5 for wild type PQQ-dependent glucose dehydrogenase is the pBluescript SK(-) vector with a structural gene encoding PQQ-dependent glucose dehydrogenase derived from *Acinetobacter baumannii* NCIMB 11517 strain inserted in the multicloning site. Its nucleotide sequence is shown in SEQ ID No. 2 in the sequence listing, while the amino acid sequence of PQQ-dependent glucose dehydrogenase as inferred from this sequence is shown as SEQ ID No. 1.

Example 2: Manufacture of modified PQQ-dependent glucose dehydrogenase

[0035] Based on recombinant plasmid pNPG5 containing the wild-type PQQ-dependent glucose dehydrogenase gene, together with the synthetic oligonucleotide shown in SEQ ID No. 3 and its complementary synthetic oligonucleotide, a mutation treatment was carried out using a QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the kit's protocols, the nucleotide sequence was then determined to obtain a recombinant plasmid (pNPGSM1) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by asparagine in the amino acid sequence shown in SEQ ID No. 2.

[0036] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 4 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M2) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by glutamic acid in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0037] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 5 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M3) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by threonine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0038] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 6 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M4) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by methionine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0039] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 7 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M5) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by glycine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0040] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 8 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M6) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 76th position is replaced by lysine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0041] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 9 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M7) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by isoleucine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

[0042] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 10 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M8) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by valine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

5 [0043] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 11 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M9) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by alanine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

10 [0044] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 22 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M10) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which lysine at 20th position is replaced by glutamic acid in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

15 [0045] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 23 and its complementary synthetic oligonucleotide, a recombinant plasmid carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which lysine at 89th position is replaced by glutamic acid in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above. Subsequently, based on the plasmid and the synthetic oligonucleotide shown in SEQ ID No. 24 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M11) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which lysine at 89th position is replaced by glutamic acid and also lysine at 300th position is replaced by arginine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

20 [0046] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 25 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M12) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 246th position is replaced by histidine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

25 [0047] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 26 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M13) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by serine and leucine at 169th position is replaced by serine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

30 [0048] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 27 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M14) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by alanine and leucine at 169th position is replaced by aspartic acid in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

35 [0049] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 66 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M15) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by serine and leucine at 169th position is replaced by glutamic acid in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

40 [0050] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 67 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M16) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by serine and leucine at 169th position is replaced by proline in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

45 [0051] Based on pNPG5 and the synthetic oligonucleotide shown in SEQ ID No. 68 and its complementary synthetic oligonucleotide, a recombinant plasmid (pNPG5M17) carrying a gene encoding modified PQQ-dependent glucose dehydrogenase in which glutamine at 168th position is replaced by alanine and leucine at 169th position is replaced by glycine in the amino acid sequence shown in SEQ ID No. 2 was obtained by methods similar to those described above.

50 [0052] *E. coli* competent cells (*Escherichia coli* JM109, Toyobo) were transformed using the recombinant plasmids pNPG5, pNPG5M1, pNPG5M2, pNPG5M3, pNPG5M4, pNPG5M5, pNPG5M6, pNPG5M7, pNPG5M8, pNPG5M9, pNPG5M10, pNPG5M11, pNPG5M12, pNPG5M13, pNPG5M14, pNPG5M15, pNPG5M16 and pNPG5M17 to obtain the respective transformants.

Example 3: Construction of expression vectors which can be replicated in *Pseudomonas* bacteria

55 [0053] 5 µg of the DNA of the recombinant plasmid pNPG5M1 obtained in Example 2 was cleaved with restriction enzymes BamHI and XhoI (Toyobo), and the structural gene area of mutated PQQ-dependent glucose dehydrogenase was isolated. The isolated DNA together with 1 µg of pTM33 cleaved with BamHI and XhoI was reacted at 16° C for 16 hours with 1 unit of T4 DNA ligase to ligate the DNAs. The ligated DNA was then transformed using competent cells



of *E. coli* DH5 $\alpha$ . The resulting expression plasmid was named PNPG6M1.

[0054] Expression plasmids were obtained by the same methods using the recombinant plasmids pNPGS, pNPG5M2, pNPG5M3, pNPG5M4, pNPG5M5, pNPG5M6, pNPG5M7, pNPG5M8, pNPG5M9, pNPG5M10, pNPG5M11, pNPG5M12, pNPG5M13, pNPG5M14, pNPG5M15, pNPG5M16 and pNPG5M17. The resulting expression plasmids were named pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5, pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16 and pNPG6M17.

Example 4: Preparation of transformant of *Pseudomonas* bacteria

[0055] *Pseudomonas putida* TE3493 (International Patent Organism Depository (IPOD) Accession No. 11298) was cultured for 16 hours at 30° C in LBG medium (LB medium + 0.3% glycerol), the cells were collected by centrifugation (12,000 rpm, 10 minutes), and 8 ml of a chilled 5 mM K-phosphate buffer (pH 7.0) containing 300 mM sucrose was added to the cells to suspend the cells. The cells were collected again by centrifugation (12,000 rpm, 10 minutes), and 0.4 ml of a chilled 5 mM K-phosphate buffer (pH 7.0) containing 300 mM sucrose was added to the cells to suspend the cells.

[0056] 0.5  $\mu$ g of the expression plasmid pNPG6M1 obtained in Example 3 was added to this suspension, and transformation was carried out by electroporation. The target transformant was obtained from a colony grown in LB agar medium containing 100  $\mu$ g/ml of streptomycin.

[0057] Transformants were obtained respectively by the same methods from the expression plasmids named pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5, pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16 and pNPG6M17.

Test Example 1

#### Measurement of GDH Activity

(1) Measurement principles

[0058]

D-glucose + PMS + PQQGDH

→ D-glucono-1,5-lactone + PMS (red)

2PMS (red) + NTB → 2PMS + Diformazan

[0059] The presence of diformazan formed by reduction of nitrotriazolium blue (NTB) with phenazine methosulfate (PMS) (red) was measured by spectrophotometry at 570 nm.

(2) Definition of units

[0060] 1 unit is defined as the amount of PQQGDH enzyme needed to form 0.5 millimoles of diformazan per minute under the conditions described below.

(3) Methods

Reagents

[0061]

A. D-glucose solution: 0.5 M (0.9 g D-glucose (molecular weight 180.16)/10 ml H<sub>2</sub>O)

B. PIPES-NaOH buffer, pH 6.5: 50 mM (1.51 g of PIPES (molecular weight 302.36) suspended in 60 ml water was dissolved in 5N NaOH, and 2.2 ml of 10% Triton X-100 was added. pH was adjusted to 6.5  $\pm$  0.05 at 25° C using 5N NaOH, and water was added to a total of 100 ml.)

C. PMS solution: 3.0 mM (9.19 mg phenazine methosulfate (molecular weight 817.65)/10 ml H<sub>2</sub>O)

D. NTB solution: 6.6 mM (53.96 mg nitrotriazolium blue (molecular weight 817.65)/10 ml H<sub>2</sub>O)

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E. Enzyme diluent: 50 mM PIPES-NaOH buffer (pH 6.5) containing 1 mM  $\text{CaCl}_2$ , 0.1% Triton X-100 and 0.1% BSA

### Procedures

5 [0062]

1. The following reaction mixture was prepared in a shaded bottle, and stored on ice (prepared when needed)

- 10 1.8 ml D-glucose solution (A)  
24.6 ml PIPES-NaOH buffer (pH 6.5) (B)  
2.0 ml PMS solution (C)  
1.0 ml NTB solution (D)

Table 1

Concentration in the assay mixture	
PIPES buffer	42 mM
D-glucose	30 mM
PMS	0.20 mM
NTB	0.22 mM

2. A plastic test tube filled with 3.0 ml of the reaction mixture was pre-heated for 5 minutes at 37°C.

3. 0.1 ml of enzyme solution was added and mixed by gentle inversion.

25 4. The increase in absorbance over water was recorded for 4-5 minutes on a spectrophotometer at 570 nm with the temperature maintained at 37° C to calculate  $\Delta\text{OD}$  per minute from the initial straight-line segment of the curve (OD test).

30 [0063] At the same time, the same methods were repeated except that enzyme diluent (E) was added in place of the enzyme solution, and the blank ( $\Delta\text{OD}$  blank) was measured.

[0064] Immediately before the assay, enzyme powder was dissolved in chilled enzyme diluent (E), and diluted to 0.1-0.8 U/ml in the same buffer (a plastic tube is preferred because of the adhesiveness of the enzyme).

### Calculations

35 [0065] Activity was calculated using the following equation:

$$\text{U/ml} = (\Delta\text{OD}/\text{min}(\Delta\text{OD test} - \Delta\text{OD blank}) \times \text{Vt} \times \text{df}) / (20.1 \times 1.0 \times \text{Vs})$$

40 
$$\text{U/mg} = (\text{U/ml}) \times 1/\text{C}$$

Vt: Total volume (3.1 ml)

Vs: Sample volume (1.0 ml)

45 20.1: 1/2 millimole molecular extinction coefficient of Difformazan

1.0: Optical path length (cm)

df: Dilution factor

C: Enzyme concentration in the solution (c mg/ml)

### 50 Preparation of Holo-expressive Purified Enzyme

[0066] 500 ml of Terrific broth was taken in a 2L volume Sakaguchi flask, autoclaved for 20 minutes at 121°C and left to cool, after which streptomycin which had been separately sterile-filtered was added to a final concentration of 100  $\mu\text{g/ml}$ . This medium was then inoculated with 5 ml of a culture solution of *Pseudomonas putida* TE3493(pNPG6M1) which had been first cultured for 24 hours at 30° C in a PY medium containing 100  $\mu\text{g/ml}$  of streptomycin, and aeration agitation cultured for 40 hours at 30°C. PQQ-dependent glucose dehydrogenase activity upon completion of the culture was about 120 U/ml per 1 ml of culture solution as measured by the methods described above.

[0067] The aforementioned cells were collected by centrifugation, suspended in a 20 mM phosphate buffer (pH 7.0),

broken up with ultrasonication and re-centrifuged. and the supernatant was taken as crude enzyme solution. This crude enzyme solution thus obtained was isolated and purified by HiTrap-SP (Amersham Pharmacia) ion exchange column chromatography. After dialysis with a 10 mM PIPES-NaOH buffer (pH 6.5). calcium chloride was added to a final concentration of 1 mM. Finally, isolation and purification were performed by HiTrap-DEAE (Amersham Pharmacia) ion exchange column chromatography to obtain the purified enzyme sample. The sample obtained by these methods exhibited a generally single SDS-PAGE band.

[0068] Purified enzyme products were also obtained by the same methods for the *Pseudomonas putida* TE3493 transformants of pNPG6, pNPG6M2, pNPG6M3, pNPG6M4, pNPG6M5, pNPG6M6, pNPG6M7, pNPG6M8, pNPG6M9, pNPG6M10, pNPG6M11, pNPG6M12, pNPG6M13, pNPG6M14, pNPG6M15, pNPG6M16 and pNPG6M17.

[0069] The resulting purified enzymes were used in evaluating properties.

#### Measurement of Km value

[0070] PQQGDH activity was measured according to the aforementioned activity measurement method. The Km value for glucose was measured by altering the substrate concentration in the aforementioned activity measurement method. The Km value for maltose was measured by substituting a maltose solution for the glucose solution in the aforementioned activity measurement method, and altering the substrate concentration as when measuring the Km value for glucose. The results are shown in Tables 2A, 2B, 6, 9 and 14.

#### Substrate Specificity

[0071] PQQGDH activity was measured according to the aforementioned activity measurement method. Using glucose as the substrate solution and maltose as the substrate solution, the respective dehydrogenase activity values were measured, and the relative value was calculated with 100 given as the measurement value using glucose as the substrate. In the case of dehydrogenase activity using maltose as the substrate solution, a 0.5M maltose solution was prepared and used for measuring activity. The results are shown in Tables 2A, 2B, 4, 5, 6, 8, 9, 11, 13 and 14.

#### Measurement of Thermal stability

[0072] The various kinds of PQQGDH were stored in buffer (10mM PIPES-NaOH (pH 6.5) containing 1mM CaCl<sub>2</sub> and 1  $\mu$ M PQQ) with an enzyme concentration of 5 U/ml, and residual activity was measured after heat treatment at 58°C. The results are shown in Tables 2A, 2B, 6, 9 and 14. The heat treatment was conducted for 20 minutes with respect to Table 2B and for 30 minutes with respect to heat treatment assays other than Table 2B.

#### Measurement of Optimum pH

[0073] Enzyme activity was measured in a 50 mM phosphate buffer (pH 5.0-8.0) containing 0.22% Triton-X100, a 50 mM acetate buffer (pH 3.0-6.0) containing 0.22% Triton-X100, a 50 mM PIPES-NaOH buffer (pH 6.0-7.0) containing 0.22% Triton-X100, and a 50 mM Tris-HCl buffer (pH 7.0-9.0) containing 0.22% Triton-X100. The results are shown in Figure 1. The pH values that produced the highest activity are given in Table 2A.

Table 2A

Mutation	Specific activity	Substrate specificity	Km (Mal)	Km (Glc)	Optimum pH	Thermal stability
Q76N	49	66%	13.6	3.1	6.4	49.1%
Q76E	36	68%	13.6	3.7	5.6	42.5%
Q76T	32	84%	10.3	2.5	6.4	49.0%
Q76M	108	81%	8.7	2.2	6.4	55.3%
Q76G	32	84%	10.6	2.2	6.4	58.5%
Q76K	84	32%	29.9	7.9	6.8	48.4%
Q168I	231	69%	11.9	5.3	6.8	27.3%

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Table 2A (continued)

Mutation	Specific activity	Substrate specificity	Km (Mal)	Km (Glc)	Optimum pH	Thermal stability
Q168V	377	71%	13.0	6.4	6.4	32.2%
Q168A	333	37%	35.3	10.4	6.4	59.2%
Wild type	1469	103%	4.1	6.5	6.4	46.7%
Notes) Specific activity: Enzyme activity(U/ml)/A280 nm absorbance (ABS) Km (Mal): Km value (mM) for maltose Km (Glc): Km value (mM) for glucose						

Table 2B

Mutation	Specific activity	Substrate specificity	Thermal stability
K20E	924	105%	49.7%
K89E + K300R	1038	81%	58.8%
Q246H	686	192%	82.2%
Q168S+L169S	288	33%	73.0%
Q168A+L169D	106	18%	78.8%
Q168S+L169E	270	19%	47.0%
Q168S+L169P	460	25%	47.2%
Q168A+L169G	170	18%	78.3%
Wild type	1469	103%	43.4%
Note) Specific activity: Enzyme activity(U/ml)/A280 nm absorbance (ABS)			

Confirmation of glucose assayability of Q76K

[0074] The following reaction reagent was prepared containing 0.45 U/ml of Q76K.

50 mM	PIPES-NaOH buffer (pH 6.5)
1 mM	CaCl <sub>2</sub>
0.22%	Triton-X100
0.4 mM	PMS
0.26 mM	WST-1 (water-soluble tetrazolium salt, Dojin Kagaku)

[0075] According to the methods described below for measuring glucose, purified water, 100 mg/dl standard solution and 10-level dilution series of aqueous glucose solution (600 mg/dl) were measured as sample, and linearity was confirmed.

[0076] The results are shown in Figure 2.

## Glucose Measurement Methods

[0077] 300  $\mu$ l of the reagent was added to 3  $\mu$ l of the sample, changes in absorbance were calculated for 1 minute beginning two minutes after addition of the reagent, and glucose levels in the samples were calculated based on a two-point calibration curve for purified water and 100 mg/dl glucose standard solution. A Hitachi 7150 automated analyzer was used as the measurement equipment, at a principle wavelength of 480 nm and a measurement temperature of 37°C.

[0078] As shown in Figure 2, good linearity was confirmed in the range of 0-600 mg/dl.

## Confirmation of Q76K Activity on Maltose

[0079] The following reaction reagent was prepared containing 0.45 U/ml of Q76K.

5

50 mM	PIPES-NaOH buffer (pH 6.5)
1 mM	CaCl <sub>2</sub>
0.22%	Triton-X100
0.4 mM	PMS
0.26 mM	WST-1 (Dojin Kagaku)

10

[0080] Samples were prepared in which 0, 120, 240 and 360 mg/dl of maltose was added to a 100 mg/dl or 300 mg/dl glucose solution. Measurements were performed according to the aforementioned measurement methods for glucose.

15

[0081] The relative values for the samples containing 100 mg/dl of glucose and various concentration of maltose were evaluated with reference to a value of 100 given for a 100 mg/dl glucose solution containing no maltose. In the same way, the relative values for the samples containing 300 mg/dl of glucose and various concentration of maltose were evaluated with reference to a value of 100 given for a 300 mg/dl glucose solution containing no maltose. The results are shown in Figure 3.

20

## Confirmation of Q76E Activity on Maltose

[0082] Activity was evaluated using Q76E in the same way as confirmation of Q76K activity on maltose. The enzyme was added at a concentration of 0.24 U/ml. The results are shown in Figure 4.

25

## Confirmation of Q168V Activity on Maltose

[0083] Activity was evaluated using Q168V in the same way as confirmation of Q76K activity on maltose. The enzyme was added at a concentration of 0.35 U/ml. The results are shown in Figure 5.

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## Confirmation of Q168A Activity on Maltose

[0084] Activity was evaluated using Q168A in the same way as confirmation of Q76K activity on maltose. The enzyme was added at a concentration of 0.6 U/ml. The results are shown in Figure 6.

35

## Confirmation of Wild-type Enzyme Activity on Maltose

[0085] Activity was evaluated using the wild-type enzyme in the same way as confirmation of Q76K activity on maltose. The enzyme was added at a concentration of 0.1 U/ml. The results are shown in Figure 7.

40

[0086] It can be seen from Figures 3, 4, 5, 6 and 7 that Q76K, Q76E, Q168V and Q168A have less activity on maltose than the wild-type enzyme.

## Example 5: Construction and screening of a mutation library

45

[0087] Using expression plasmid pNPG5 as the template, random mutations were introduced into the 167-169 region of the structural gene by PCR. The PCR reaction was performed in a solution of the composition shown in Table 3 for 2 minutes at 98° C, followed by 30 cycles of 20 seconds at 98° C, 30 seconds at 60° C and 4 minutes at 72° C.

Table 3

50

Reagent	Liquid volume
KOD Dash DNA Polymerase (2.5 U/ $\mu$ l)	1.0 $\mu$ l
Template DNA	1.0 $\mu$ l
Forward primer (SEQ ID No. 12)	2.5 $\mu$ l
Reverse primer (SEQ ID No. 13)	2.5 $\mu$ l
10 $\times$ buffer	5.0 $\mu$ l
2 mM dNTPs	5.0 $\mu$ l

55

Table 3 (continued)

Reagent	Liquid volume
H <sub>2</sub> O	33.0 µl

[0088] The resulting mutation library was transformed into *E. coli* DH5α, and the formed colonies were transplanted to microtitre plates filled with 180 µl/well of LB medium (containing 100 µg/ml of ampicillin and 26 µM of PQQ) and cultured for 24 hours at 37°C. 50 µl of each culture liquid was transferred to a separate microtitre plate, and the cultured cells were broken down by repeated freezing and thawing and centrifuged (2,000 rpm, 10 minutes) to collect the supernatant. Two microtitre plates were filled with 10 µl each of the collected supernatant. One microtitre plate was measured for activity using an activity measuring reagent with glucose as the substrate, and the other microtitre plate was measured for activity using an activity measurement reagent having maltose as the substrate, and the reactions were compared. Several clones were obtained having altered reactivity with respect to maltose.

[0089] Those clones having altered reactivity on maltose were cultured in test tubes filled with 5 ml of LB medium (containing 100 µg/ml of ampicillin and 26 µM of PQQ), and confirmation tests showed several of the clones having altered reactivity with respect to maltose.

[0090] The results are shown in Table 4.

Table 4

Mutation Site	Activity on Maltose	Mutation Site	Activity on Maltose
N167E+Q168G+L169T	64%	N167S+Q168N+L169R	80%
Q168G+L169T	42%	N167G-Q168S+L169Y	55%
N167L+Q168S+L169G	45%	N167G+Q168S+L169S+ L174F+K49N	39%
Q168N+L169N+S189R	51%	N167E+Q168G+L169A+ S189G	58%
N167G+Q168R+L169A	66%	N167S+Q168G+L169A	48%
N167G+Q168V+L169S	42%	N167S+Q168V+L169S	71%
N167T+Q168I+L169G	42%	N167G+Q168W+L169N	72%
N167G+Q168S+L169N	50%	N167G+Q168S+L169V	36%
Q168R+L169C	29%	N167S+Q168L+L169G	41%
Q168C+L169S	33%	N167T+Q168N+L169K	68%
N167G+Q168T+L169A +S207C	24%	N167A+Q168A+L169P	63%
N167G+Q168S+L169G	34%	N167G+Q168G	46%
N167G+Q168D+L169K	35%	Q168P+L169G	23%
N167G+Q168N+L169S	59%	Q168S+L169G	22%
N188I+T349S	64%	N167G+Q168G+L169A+ F215Y	32%
N167G+Q168T+L169G	28%	Q168G+L169V	43%
N167G+Q168V+L169T	43%	N167E+Q168N+L169A	52%
Q168R+L169A	72%	N167G+Q168R	23%
N167G+Q168T	69%	N167G+Q168T+L169Q	72%
Q168I+L169G+K300T	24%	N167G+Q168A	33%
N167T+Q168L+L169K	63%	N167M+Q168Y+L169G	60%
N167E+Q168S	32%	N167G+Q168T+L169V+ S189G	42%
N167G+Q168G+L169C	37%	N167G+Q168K+L169D	41%
Q168A+L169D	16%	Q168S+E245D	29%
Q168S+L169S	26%	A351T	74%

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Table 4 (continued)

Mutation Site	Activity on Maltose	Mutation Site	Activity on Maltose
N167S+Q168S+L169S	51%	Q168I+L169Q	51%
N167A+Q168S+L169S	40%	Q168A	35%
Q168S+L169P	20%	Q168A+L169G	16%
Q168S+L169E	15%		
+			

[0091] Mutations were also introduced in the same way in regions 67-69 (forward primer: SEQ ID No. 14, reverse primer: SEQ ID No. 15), 129-131 (forward primer: SEQ ID No. 16, reverse primer: SEQ ID No. 17), 341-343 (forward primer: SEQ ID No. 18, reverse primer: SEQ ID No. 19). An insertion was also attempted between positions 428 and 429 (forward primer: SEQ ID No. 20, reverse primer: SEQ ID No. 21). The results are shown in Table 5.

Table 5

67-69 Region			
Mutation site	Activity on maltose	Mutation site	Activity on maltose
P67K+E68K	79%	P67R+E68R+I6 9C	80%
P67D+E68T+I6 9C	60%		
129-131 Region			
Mutation site	Activity on maltose	Mutation site	Activity on maltose
E129R+K130G+P1 31G	73%	E129Q+K130T+P1 31R	80%
E129N+P131T	67%	E129A+K130R+P1 31K	70%
341-343 Region			
Mutation site	Activity on maltose	Mutation site	Activity on maltose
E341L+M342P+A3 43R	80%	E341S+M342I	80%
A343I	45%	E341P+M342V+A3 43C	50%
E341P+M342V+A3 43R	76%	E341L+M342R+A3 43N	51%
Insertion between 428 and 429			
Inserted amino acid	Activity on maltose	Inserted amino acid	Activity on maltose
L	73%	A	71%
K	79%		

[0092] Those mutants with greatly reduced activity on maltose were selected (Q168S + E245D, Q168A + L169D, Q168S + L169S, Q168S + L169E, Q168A + L169G, Q168S + L169P), plasmids were extracted from these mutants, *Pseudomonas* was transformed according to the methods described in Examples 3 and 4 to express holoenzymes, and purified enzymes were obtained and their properties were evaluated. The results are shown in Table 6.

Table 6

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168S+E245D	714	29%	24.3	14.4	55.5%
Q168A+L169D	106	18%	65.9	20.8	89-4%
Q168S+L169S	288	33%	55.1	14.4	83.9%
Q168S+L169P	460	25%	87.1	24.1	76.3%
Q168A+L169G	170	18%	60.4	18.6	89.5%

Table 6 (continued)

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168S+L169E	270	19%	70.7	8.9	63.3%
Q168A	313	43%			64.4%
Wild type	1469	110%			59.8%
Note) Specific activity: Enzyme activity(U/ml)/A280 nm absorbance (ABS)					

Example 6: Effect of mutation at Q168 site on substrate specificity

[0093] Mutants such as Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168P, Q168R, Q168S, Q168T, Q168W and Q168Y were prepared according to the methods disclosed in example 5. Primers used for preparing the mutants are shown in table 7. Further, Table 8 shows the results comparing reactivity of the mutants on maltose by using each mutant in the form of broken cells which were prepared by test tube culture. Mutant enzymes were obtained by extracting plasmids from the mutants, followed by transforming *Pseudomonas* according to the methods described in Examples 3 and 4 to express holoenzymes. The properties of purified enzymes were evaluated. The results are shown in table 9.

Table 7

Mutation site	Forward primer	Reverse primer
Q168C	SEQ ID No.22	SEQ ID No.23
Q168D	SEQ ID No.22	SEQ ID No.24
Q168E	SEQ ID No.22	SEQ ID No.25
Q168F	SEQ ID No.22	SEQ ID No.26
Q168G	SEQ ID No.22	SEQ ID No.27
Q168H	SEQ ID No.22	SEQ ID No.28
Q168K	SEQ ID No.22	SEQ ID No.29
Q168L	SEQ ID No.22	SEQ ID No.30
Q169M	SEQ ID No.22	SEQ ID No.31
Q168N	SEQ ID No.22	SEQ ID No.32
Q168P	SEQ ID No.22	SEQ ID No.33
Q168R	SEQ ID No.22	SEQ ID No.34
Q168S	SEQ ID No.22	SEQ ID No.35
Q168T	SEQ ID No.22	SEQ ID No.36
Q168W	SEQ ID No.22	SEQ ID No.37
Q168Y	SEQ ID No.22	SEQ ID No.38

Table 8

Mutation site	Activity on Maltose	Mutation site	Activity on Maltose
Q168C	54%	Q169M	64%
Q168D	29%	Q168N	82%
Q168E	36%	Q168P	103%
Q168F	43%	Q168R	36%
Q168G	46%	Q168S	60%



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Table 8 (continued)

Mutation site	Activity on Maltose	Mutation site	Activity on Maltose
Q168H	55%	Q168T	94%
Q168K	83%	Q168W	87%
Q168L	92%	Q168Y	93%
Wild type	104%		

Table 9

Mutation	Specific activity	Substrate specificity )	Km(Mal	Km(Glc )	Thermal stability
Q168C	55	58%	20.4	10.7	18.2%
Q168D	102	46%	27.4	-	61.4%
Q168E	110	51%	4.7	8.6	75.4%
Q168F	137	52%	36.4	10.3	55.5%
Q168G	667	78%	11.1	-	78.7%
Q168H	486	58%	10.2	5.4	76.0%
Q168K	5	80%	9.6	2.2	-
Q168L	110	96%	8.6	4.3	37.1%
Q169M	190	68%	22.7	5.3	78.4%
Q168N	68	93%	3.6	4.1	-
Q168P	128	106%	3.5	5.1	82.3%
Q168R	57	60%	18.4	3.8	32.9%
Q168S	483	81%	12.5	3.7	80.1%
Q168T	11	103%	15.0	6.9	-
Q168W	287	96%	5.3	3.2	59.2%
Q168Y	297	99%	12.1	6.9	100.0%
Wild type	1285	106%	3.8	6.3	52.2%
Note) Specific activity: Enzyme activity(U/ml)/A280 nm absorbance (ABS)					

Example 7: Effect of mutation at L169 site on substrate specificity

[0094] Mutants such as L169A, L169V, L169H, L169Y, L169K, L169D, L169S, L169N, L169G and L169C were prepared according to the methods disclosed in example 2. Primers used for preparing the mutants are shown in table 10. Further, table 11 shows the results comparing reactivity of the mutants on maltose by using each mutant in the form of broken cells which were prepared by test tube culture.

Table 10

Mutation site	Forward primer	Reverse primer
L169A	SEQ ID No.39 Synthetic	oligonucleotide complementary to SEQ ID No.39
L169V	SEQ ID No.40	Synthetic oligonucleotide complementary to SEQ ID No.40
L169Y	SEQ ID No.41	Synthetic oligonucleotide complementary to SEQ ID No.41
L169H	SEQ ID No.42	Synthetic oligonucleotide complementary to SEQ ID No.42
L169K	SEQ ID No.43	Synthetic oligonucleotide complementary to SEQ ID No.43

Table 10 (continued)

Mutation site	Forward primer	Reverse primer
L169D	SEQ ID No.44	Synthetic oligonucleotide complementary to SEQ ID No.44
L169S	SEQ ID No.45	Synthetic oligonucleotide complementary to SEQ ID No.45
L169N	SEQ ID No.46	Synthetic oligonucleotide complementary to SEQ ID No.46
L169G	SEQ ID No.47	Synthetic oligonucleotide complementary to SEQ ID No.47
L169C	SEQ ID No.48	Synthetic oligonucleotide complementary to SEQ ID No.48

Table 11

Mutation site	Activity on Maltose	Mutation site	Activity on Maltose
L169A	59%	L169D	38%
L169V	78%	L169S	57%
L169Y	107%	L169N	74%
L169H	85%	L169G	48%
L169K	60%	L169C	57%
Wild type	97%		

Example 8: Effect of combination of mutations of Q168A together with L169 site on substrate specificity

[0095] Mutants such as Q168A+L169A, Q168A+L169C, Q168A+L169E, Q168A+L169F, Q168A+L169H, Q168A+L169I, Q168A+L169K, Q168A+L169M, Q168A+L169N, Q168A+L169P, Q168A+L169Q, Q168A+L169R, Q168A+L169S, Q168A+L169T, Q168A+L169V, Q168A+L169W, Q168A+L169Y were prepared according to the methods disclosed in example 5. Primers used for preparing the mutants are shown in table 12. Further, Table 13 shows the results comparing reactivity of the mutants on maltose by using each mutant in the form of broken cells which were prepared by test tube culture. Mutant enzymes were obtained by extracting plasmids from the mutants, followed by transforming *Pseudomonas* according to the methods described in Examples 3 and 4 to express holoenzymes. The properties of purified enzymes were evaluated. The results are shown in table 14.

Table 12

Mutation site	Forward primer	Reverse primer
Q168A+L169A	SEQ ID No. 12	SEQ ID No.49
Q168A+L169C	SEQ ID No.12	SEQ ID No.50
Q168A+L169E	SEQ ID No. 12	SEQ ID No.51
Q168A+L169F	SEQ ID No.12	SEQ ID No.52
Q168A+L169H	SEQ ID No.12	SEQ ID No.53
Q168A+L169I	SEQ ID No. 12	SEQ ID No.54
Q168A+L169K	SEQ ID No.12	SEQ ID No.55
Q168A+L169M	SEQ ID No. 12	SEQ ID No.56
Q168A+L169N	SEQ ID No.12	SEQ ID No.57
Q168A+L169P	SEQ ID No.12	SEQ ID No.58
Q168A+L169Q	SEQ ID No.12	SEQ ID No.59
Q168A+L169R SEQ	ID No.12	SEQ ID No.60
Q168A+L169S	SEQ ID No.12	SEQ ID No.61
Q168A+L169T	SEQ ID No.12	SEQ ID No.62

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Table 12 (continued)

Mutation site	Forward primer	Reverse primer
Q168A+L169V	SEQ ID No.12	SEQ ID No.63
Q168A+L169W	SEQ ID No.12	SEQ ID No.64
Q168A+L169Y	SEQ ID No.12	SEQ ID No.65

Table 13

Mutation site	Activity on maltose	Mutation site	Activity on maltose
Q168A+L169A	19%	Q168A+L169P	24%
Q168A+L169C	7%	Q168A+L169Q	42%
Q168A+L169E	17%	Q168A+L169R	42%
Q168A+L169F	22%	Q168A+L169S	14%
Q168A+L169H	21%	Q168A+L169T	24%
Q168A+L169I	43%	Q168A+L169V	34%
Q168A+L169K	21%	Q168A+L169W	33%
Q168A+L169M	22%	Q168A+L169Y	37%
Q168A+L169N	19%	Wild type	104%

Table 14

Mutation	Specific activity	Substrate specificity	Km(Mal)	Km(Glc)	Thermal stability
Q168A+L169A	154	19%	126	33.0	86.2%
Q168A+L169C	63	13%	103	35.6	100.0%
Q168A+L169E	90	19%	8.6	20.4	100.0%
Q168A+L169F	138	27%	44.7	10.4	80.4%
Q168A+L169H	70	27%	99.2	15.5	100.0%
Q168A+L169I	43	53%	12.5	6.0	28.7%
Q168A+L169K	129	20%	20.4	26.7	100.0%
Q168A+L169M	80	23%	52.3	15.6	-
Q168A+L169N	167	22%	59.1	34.5	83.5%
Q168A+L169P	377	24%	58.0	13.9	79.9%
Q168A+L169Q	117	49%	156.9	5.4	100.0%
Q168A+L169R	32	45%	59.0	9.6	100.0%
Q168A+L169S	42	24%	15.6	21.0	-
Q168A+L169T	98	23%	33.5	15.2	83.7%
Q168A+L169V	41	27%	49.1	24.7	40.4%
Q168A+L169W	91	38%	63.3	10.8	49.4%
Q168A+L169Y	31	52%	13.6	11.6	74.3%
Wild type	1285	106%	3.8	6.3	52.2%

Note) Specific activity: Enzyme activity(U/ml)/A280 nm absorbance (ABS)

[0096] With the present invention it is possible to obtain PQQGDH having improved substrate specificity and thermal

stability.

## SEQUENCE LISTING

5 [0097]

&lt;110&gt; TOYO BOSEKI KABUSHIKI KAISHA

10 <120> MODIFIED PYRROLOQUINOLINE QUINONE (PQQ) DEPENDENT GLUCOSE  
DEHYDROGENASE WITH SUPERIOR SUBSTRATE SPECIFICITY AND STABILITY

&lt;130&gt; 20033AEP

15 &lt;160&gt; 68

&lt;170&gt; PatentIn version 3.1

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36

## Claims

1. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) having less activity on disaccharides and/or greater stability than wild-type PQQGDH.
2. The modified PQQGDH according to claim 1, wherein activity on disaccharides is less than that of wild-type glucose dehydrogenase.
3. The modified PQQGDH according to claim 2, wherein the activity on maltose is no more than 90% of that on glucose.
4. The modified PQQGDH according to claim 2, wherein the Km value for disaccharides is increased.
5. The modified PQQGDH according to claim 4, wherein the Km value for maltose is 8 mM or greater.
6. The modified PQQGDH according to claim 2, wherein the Km value for disaccharides is greater than that for glucose.
7. The modified PQQGDH according to claim 2, 4 or 6, wherein the disaccharide is maltose.
8. The modified PQQGDH according to claim 6, wherein the Km value for maltose is at least 1.5 times the Km value for glucose.
9. The modified PQQGDH according to claim 2, wherein amino acids involved in glucose binding and/or surrounding amino acids are substituted in the PQQ-dependent glucose dehydrogenase shown in SEQ ID NO:1.
10. The modified PQQGDH according to claim 2, wherein one or more amino acids at positions selected from the group consisting of positions 67, 68, 69, 76, 89, 167, 168, 169, 341, 342, 343, 351, 49, 174, 188, 189, 207, 215, 245, 300, 349, 129, 130 and 131 are substituted in the PQQ-dependent glucose dehydrogenase shown in SEQ ID NO:1.
11. The modified PQQGDH according to claim 10, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, N167E, N167L, N167G, N167T, N167S, N167A, N167M, Q1681, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169D, L169S, L169W, L169Y, L169A, L169N, L169M, L169V, L169C, L169Q, L169H, L169F, L169R, L169K, L169I, L169T, K89E, K300R, S207C, N1881, T349S, K300T, L174F, K49N, S189G, F215Y, S189G, E245D, A351T, P67K, E68K, P67D, E68T, 169C, P67R, E68R, E129R, K130G, P131G, E129N, P131T, E129Q, K130T, P131R, E129A, K130R, P131K, E341L, M342P, A343R, A343I, E341P, M342V, E341S, M342I, A343C, M342R, A343N, L169P, L169G and L169E.
12. The modified PQQGDH according to claim 10, wherein the amino acid substitution is selected from the group consisting of Q76N, Q76E, Q76T, Q76M, Q76G, Q76K, Q1681, Q168V, Q168A, Q168C, Q168D, Q168E, Q168F, Q168G, Q168H, Q168K, Q168L, Q168M, Q168N, Q168R, Q168S, Q168W, L169A, L169V, L169H, L169K, L169D, L169S, L169N, L169G, L169C, (K89E+K300R), (Q168A+L169D), (Q168S+L169S), (N167E+Q168G+L169T),

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13. The modified PQQGDH according to claim 2, wherein an amino acid is inserted between positions 428 and 429 in the PQQ-dependent glucose dehydrogenase shown in SEQ ID NO:1.
14. A modified pyrroloquinoline quinone dependent glucose dehydrogenase (PQQGDH) wherein stability is improved over that of wild-type PQQGDH.
15. The modified PQQGDH according to claim 14, wherein residual activity after heat treatment at 58°C for 30 minutes is 48% or more, 55% or more or 70% or more.
16. The modified PQQGDH according to claim 14, wherein one or more amino acids at positions selected from the group consisting of positions 20, 76, 89, 168, 169, 246 and 300 are substituted in the PQQGDH shown in SEQ ID NO:1.
17. The modified PQQGDH according to claim 16, wherein the amino acid substitutions are selected from the group of K20E, Q76M, Q76G, K89E, Q168A, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, L169D, L169E, L169P, L169S, Q246H, K300R, Q76N, Q76T, Q76K, L169A, L169C, L169E, L169F, L169H, L169K, L169N, L169Q, L169R, L169T, L169Y and L169G.
18. The modified PQQGDH with improved thermal stability according to claim 17, wherein the amino acid substitutions are selected from the group of K20E, Q76M, Q76G, (K89E+K300R), Q168A, (Q168A+L169D), (Q168S+L169S), Q246H, Q168D, Q168E, Q168F, Q168G, Q168H, Q168M, Q168P, Q168W, Q168Y, Q168S, ((Q168S+L169E), (Q168S+L169P), (Q168A+L169A), (Q168A+L169C), (Q168A+L169E), (Q168A+L169F), (Q168A+L169H), (Q168A+L169K), (Q168A+L169N), (Q168A+L169P), (Q168A+L169Q), (Q168A+L169R), (Q168A+L169T) and (Q168A+L169Y) and (Q168A+L169G).
19. A gene coding for the modified PQQGDH according to any one of claims 1 to 18.
20. A vector containing the gene according to claim 19.
21. A transformant transformed by the vector according to claim 20.
22. A method of manufacturing a modified PQQGDH, comprising cultivating the transformant according to claim 21.
23. A glucose assay kit comprising the modified PQQGDH according to any one of claims 1 to 22.
24. A glucose sensor comprising the modified PQQGDH according to any one of claims 1 to 22.
25. A method for determining glucose concentration in a sample using the modified PQQGDH according to any one of claims 14 to 21.



26. The modified PQQGDH according to claim 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 15 Å from an active center of three-dimensional active structure of wild-type enzyme.
- 5 27. The modified PQQGDH according to claim 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a substrate in three-dimensional active structure of wild-type enzyme-substrate complex.
- 10 28. The modified PQQGDH according to claim 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a OH group bound to a carbon at position 1 of a substrate in a three-dimensional active structure of wild-type enzyme-substrate complex.
- 15 29. The modified PQQGDH according to claim 1, which is obtained by mutation of at least one amino acid located within a range having a radius of 10 Å from a OH group bound to a carbon at position 2 of a substrate in a three-dimensional active structure of wild-type enzyme-substrate complex.
30. The modified PQQGDH according to any one of claims 27 to 29, whose substrate is glucose.

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Fig. 1

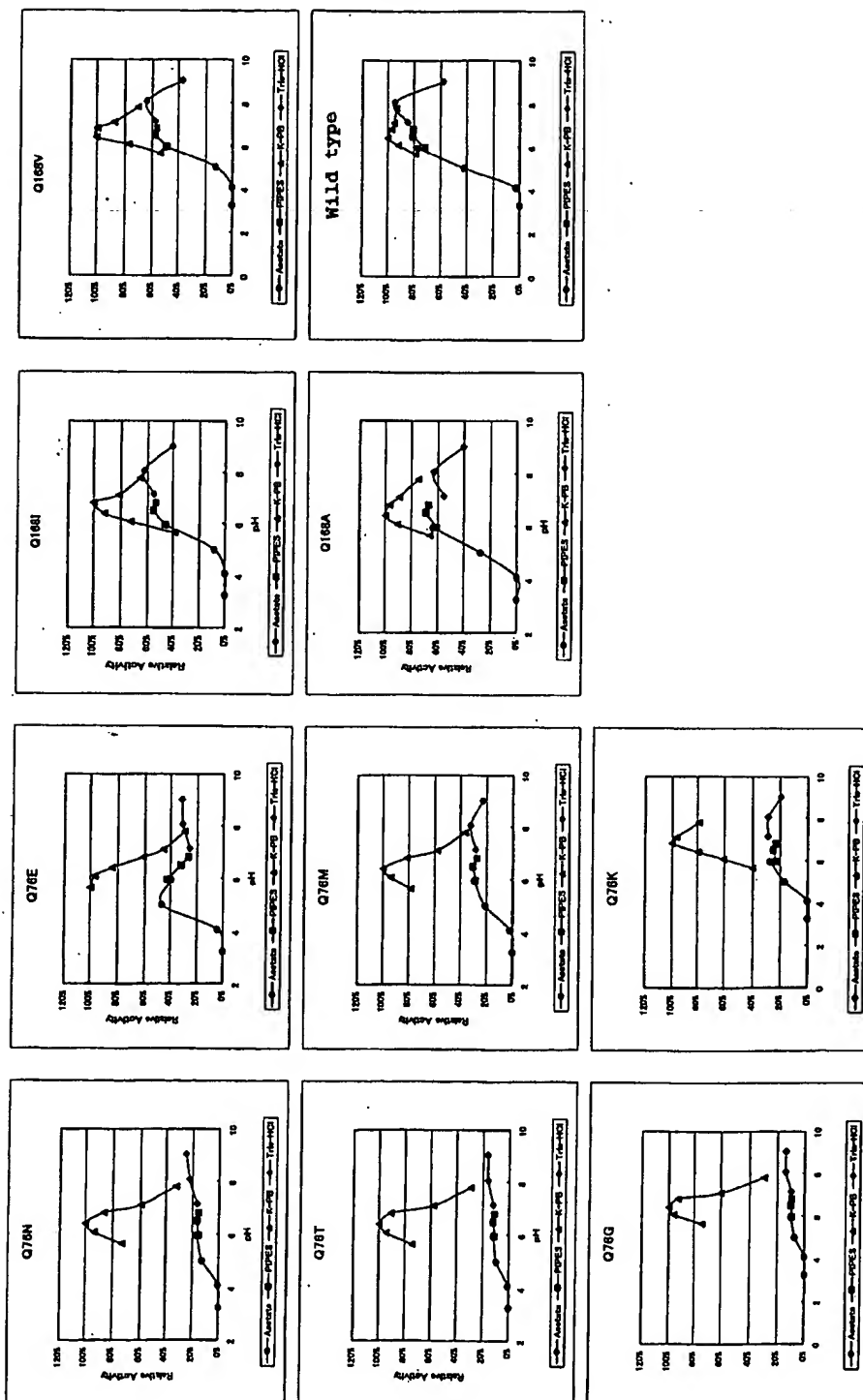


Fig. 2

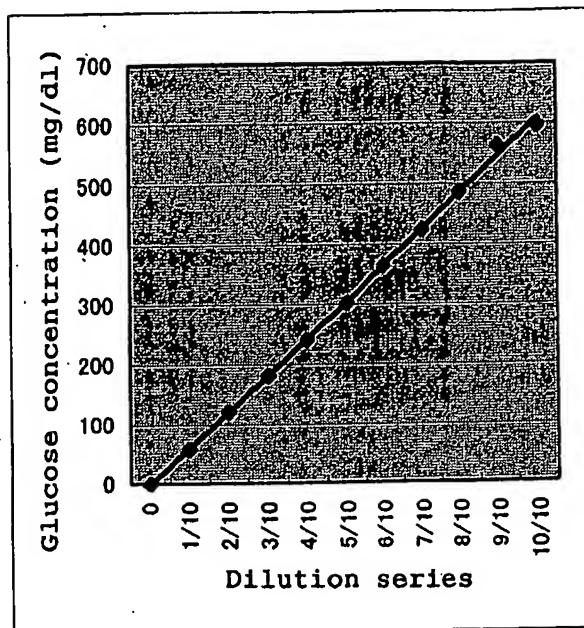


Fig. 3

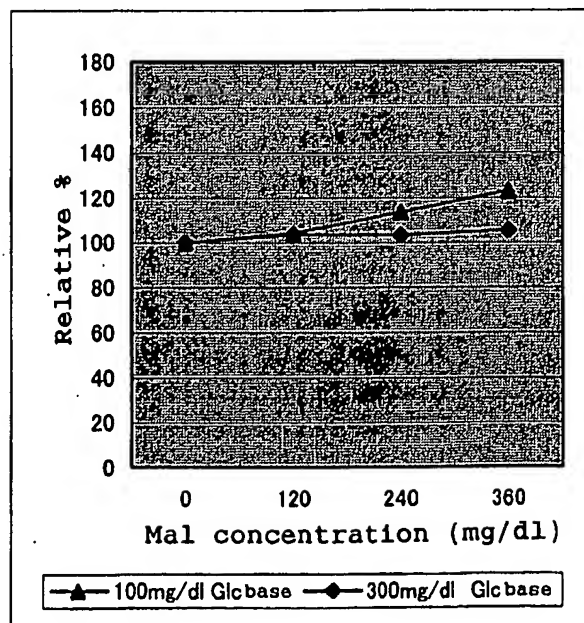


Fig. 4

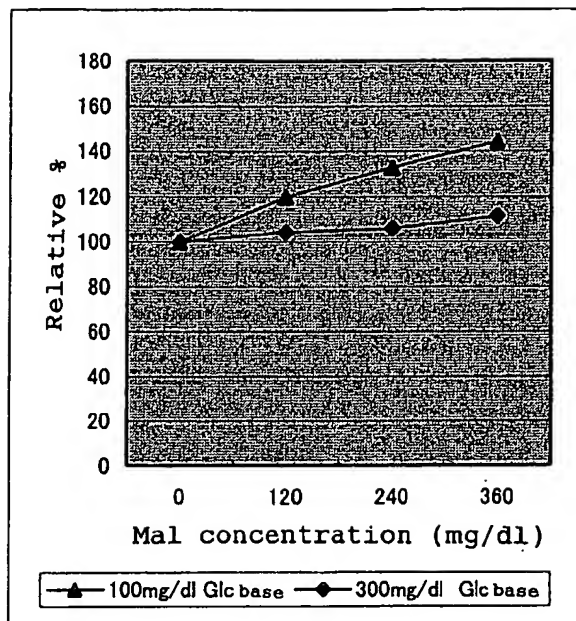


Fig. 5

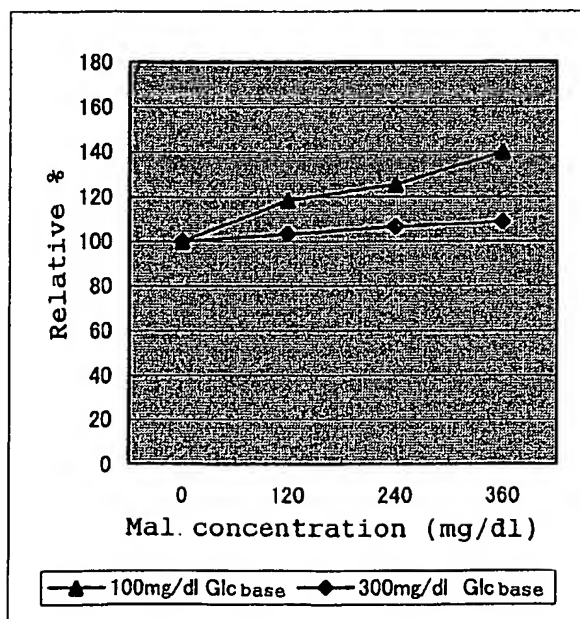


Fig. 6

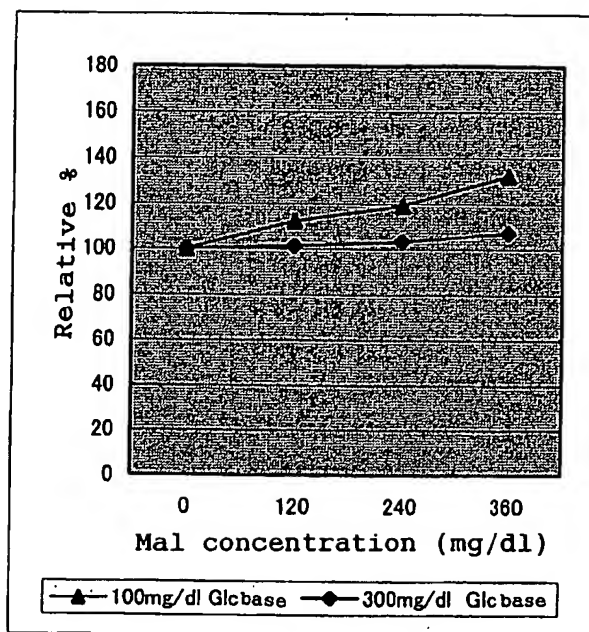
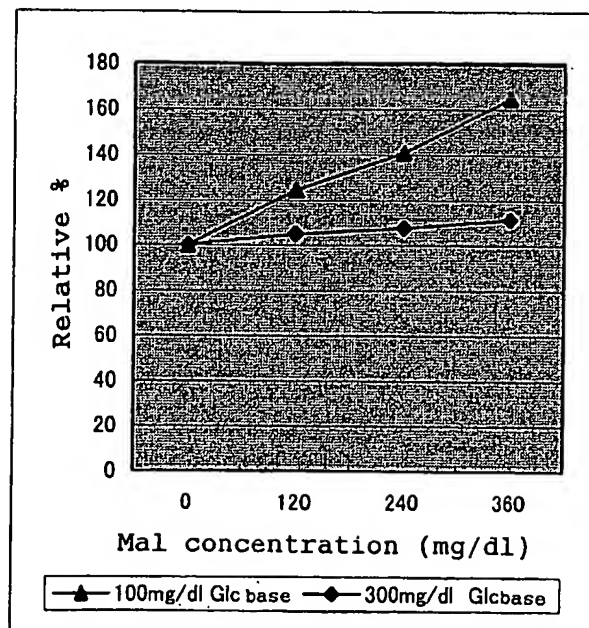


Fig. 7



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